

# Genetic Evidence Supporting Selection of the V $\alpha$ 14i NKT Cell Lineage from Double-Positive Thymocyte Precursors

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## Summary

Invariant V $\alpha$ 14i NKT (iNKT) cells are a specialized subset of T lymphocytes with regulatory functions. They coexpress TCR $\alpha\beta$  and natural killer cell markers. They differentiate through interaction of their V $\alpha$ 14-J $\alpha$ 18 invariant TCR $\alpha$  chains with CD1d expressed on double-positive (DP) thymocytes. Although their development has been shown to be thymus dependent, their developmental pathway has not been definitively established. By using genetic analyses, we show here that all iNKT cells are selected from a pool of DP thymocytes. Their development is absolutely dependent on Runx1 and ROR $\gamma$ t, transcription factors that influence, but are not required for, development of conventional T cells. Our results indicate that even though CD1d binding DP thymocytes have yet to be observed, V $\alpha$ 14-J $\alpha$ 18 rearrangement in these cells is required for development of iNKT cells.

## Introduction

T lymphocyte development proceeds after migration into the thymus of bone marrow-derived common lymphoid progenitors (Kondo et al., 1997). Conventional T cells that express TCR $\alpha\beta$  have a diverse repertoire due

to random rearrangement of TCR V(D)J elements followed by appropriate selection (von Boehmer et al., 2003). At the CD4<sup>+</sup>8<sup>+</sup> DP stage, thymocytes expressing TCR $\alpha\beta$  with appropriate avidity for self-MHC-peptide complexes are selected through positive and negative selection upon interaction with MHC class I or class II molecules expressed on thymic epithelial cells and undergo maturation to CD4<sup>+</sup>8<sup>−</sup> or CD4<sup>+</sup>8<sup>+</sup> single-positive thymocytes.

V $\alpha$ 14 invariant NKT cells (iNKTs) are a subset of TCR $\alpha\beta$  cells characterized by expression of a semi-invariant V $\alpha$ 14-J $\alpha$ 18 TCR $\alpha$  chain and markers traditionally associated with the NK cell lineage, including NK1.1 and members of the Ly-49 family of molecules (Kronenberg and Gapin, 2002; MacDonald, 2002). These cells are thought to function as regulatory cells by producing large amounts of cytokines, and defective iNKT cell development has been linked to autoimmunity (Lehuen et al., 1998; Poulton et al., 2001). Development of iNKT cells is dependent on a nonclassical MHC molecule, CD1d, expressed on DP thymocytes (Bendelac, 1995; Coles and Raulet, 2000). iNKT cells were recently shown to recognize a lysosomal sphingolipid as an endogenous ligand (Zhou et al., 2004b). A synthetic glycolipid from marine sponge,  $\alpha$ -galactosyl ceramide ( $\alpha$ GC), functions as a specific agonist for the invariant TCRs when it is presented in the context of CD1d (Burdin et al., 1999; Kawano et al., 1997). In recent years, development of fluorescently labeled recombinant CD1d tetramer loaded with  $\alpha$ GC has allowed ready detection of iNKT cells and identification of immature iNKT cell precursors that lack expression of the NK markers (Benlagha et al., 2002). These studies have shown that iNKT cell precursors undergo robust proliferation before they express NK1.1 and that their survival requires NF- $\kappa$ B activation (Benlagha et al., 2002; Stanic et al., 2004b). In more mature stages, iNKT cells require the transcription factor T-bet and cytokine signaling initiated by interleukin-15 (IL-15) (Kennedy et al., 2000; Lodolce et al., 1998; Townsend et al., 2004). Lymphotoxin beta receptor (LT $\beta$ -R), the Src family tyrosine kinase Fyn, and Ets family transcription factors also contribute to iNKT cell development, although it is not clear whether these factors are required at the early or late stages (Eberl et al., 1999; Elewaut et al., 2000; Iizuka et al., 1999; Lacorazza et al., 2002; Walunas et al., 2000).

Although iNKT cells represent a distinct lineage of TCR $\alpha\beta$  cells, their developmental relationship to conventional TCR $\alpha\beta$  cells remains unclear. Two models have been proposed for iNKT cell development. The “precommitment model” proposes that a deterministic mechanism dictates a unique maturation program for the iNKT cell lineage before positive selection (Ballas et al., 1997; Iwabuchi et al., 2001; Sato et al., 1999). According to this model, iNKT cell precursors may or may not pass through DP thymocytes. The “TCR-instructive model,” which is also known as the “mainstream model,” proposes that iNKT cells are derived from a common precursor pool shared with conven-

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tional TCR $\alpha\beta$  cells in which cells undergo random TCR $\alpha$  rearrangements and are selected to the iNKT cell lineage as a consequence of a specific rearrangement for the invariant TCR $\alpha$  chain (Bendelac et al., 1996). According to this model, referred to here as the selection model, iNKT cells are derived from DP thymocytes, and their fate is determined at the DP stage. A recent study identified canonical V $\alpha$ 14-J $\alpha$ 18 rearrangements in large DP thymocytes, and it was shown earlier that intra-thymically transferred DP thymocytes could give rise to iNKT cells in irradiated recipients (Dao et al., 2004; Gapin et al., 2001). The latter study also showed that CD1d<sup>-/-</sup> DP thymocytes could give rise to iNKT cells when transferred into CD1d-expressing hosts. The authors concluded that iNKT cells are derived from DP thymocytes, and their findings supported the selection model. However, this study did not rule out the possibility that rare contaminating cells in the donor DP thymocyte preparation gave rise to iNKT cells in the transfer experiments. Because iNKT cell precursors undergo extensive proliferation during their maturation (Benlagha et al., 2002), a very small number of contaminating cells could give rise to a substantial population of CD1d-tetramer-positive cells in the recipients. In addition, even if DP cells are precursors for iNKT cells, they may give rise to only a fraction of the mature iNKT cell population. Thus, it is still unclear whether all iNKT cells are derived from DP thymocytes and whether they differentiate from precommitted thymocytes or as a consequence of selection.

Runx family members and ROR $\gamma$ t are transcription factors that have key functions during thymocyte differentiation (Sun et al., 2000; Taniuchi et al., 2002). Runx1 and Runx3 differentially regulate CD4 silencing at discrete stages of thymocyte differentiation (Taniuchi et al., 2002). In the absence of Runx1, thymocyte selection and development of mature CD4 and CD8 TCR $\alpha\beta$  cells are reduced (Taniuchi et al., 2002). ROR $\gamma$ t regulates survival of DP thymocytes and is essential for secondary lymphoid organogenesis (Sun et al., 2000). In thymus, it is specifically expressed in DP thymocytes, where it induces expression of the antiapoptotic factor Bcl-xL and, thus, promotes survival and consequent extensive TCR $\alpha$  locus rearrangement (Guo et al., 2002; Sun et al., 2000). ROR $\gamma$ t is not essential for development of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, although, in ROR $\gamma$ t-deficient mice, these cells are reduced in number and have a TCR $\alpha$  repertoire that reflects rearrangement of proximal V $\alpha$  and J $\alpha$  segments (Guo et al., 2002).

In this study, we have used a fate-mapping strategy to demonstrate that iNKT cells are derived from DP thymocytes in which ROR $\gamma$ t/Bcl-xL-dependent V $\alpha$ 14 to J $\alpha$ 18 TCR $\alpha$  rearrangement permits specification of the iNKT cell fate. The precursor-intrinsic requirement for both ROR $\gamma$ t and Runx1 in DP thymocytes is most consistent with a selection mechanism in the developmental pathway of iNKT cells.

## Results

### NKT Cells Are Derived from DP Thymocytes

To determine the origin of iNKT cells, we performed fate-mapping experiments with DP stage-specific Cre transgenic mice that were bred to ROSA26R reporter

mice. We first used CD4-cre transgenic mice for the fate mapping of iNKT cells. In this transgenic strain, the Cre recombinase is expressed in DP thymocytes and in CD4 single-positive T cells under the control of the proximal enhancer and the silencer of the CD4 gene (Sawada et al., 1994). Therefore, deletion of a loxP-flanked sequence occurs in DP thymocytes. We bred the CD4-cre transgenic mice to the ROSA26R reporter strain, in which excision of the loxP-flanked “stop” sequence turns on EGFP reporter gene expression, and traced fates of DP thymocyte-derived cells in lymphoid tissues. As shown in Figure 1A, CD4<sup>+</sup>TCR $\beta$ <sup>+</sup> cells and CD8<sup>+</sup>TCR $\beta$ <sup>+</sup> cells from lymph nodes of the double-transgenic mice expressed the EGFP reporter, whereas EGFP expression was not detected in TCR $\beta$ <sup>-</sup> cells. In a previous study, we showed that TCR $\gamma\delta$  cells are not labeled in this strain of transgenic mice (Eberl and Littman, 2004). These findings confirm that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are derived from DP thymocytes whose progeny are genetically labeled by EGFP. Next, EGFP expression in iNKT cells in the thymus and liver was examined in the double-transgenic mice (Figures 1B and 1C and see Figure S1 in the Supplemental Data available with this article online). CD1d-restricted iNKT cells, defined by binding of CD1d- $\alpha$ GC tetramer, showed uniform expression of the EGFP reporter both in the thymus and liver. Notably, CD1d- $\alpha$ GC<sup>+</sup> cells, including NK1.1<sup>-</sup> immature iNKT cells or CD4<sup>-</sup>CD8<sup>-</sup> iNKT cells, were EGFP positive (Figure 1B and data not shown). Staining with anti-NK1.1 and anti-TCR $\beta$  also showed that NK1.1<sup>+</sup> TCR $\beta$ <sup>+</sup> cells in the thymus and liver were GFP positive (Figure S1). These results demonstrate that iNKT cells are likely to be derived from DP thymocytes.

Because a substantial proportion of iNKT cells is CD4 positive and this CD4 gene activity may allow them to express EGFP even if they do not differentiate from DP thymocytes, we used another Cre transgenic strain that expresses the cre transgene under the control of the Rorc( $\gamma$ t) (ROR $\gamma$ t) gene (Eberl and Littman, 2004). To carefully examine ROR $\gamma$ t expression in the thymus before the fate mapping, we first analyzed the expression of EGFP in thymocytes including the iNKT populations from ROR $\gamma$ t-EGFP knockin mice (Rorc( $\gamma$ t)<sup>gfp/+</sup>) (Eberl et al., 2004) (Figure 2A). ROR $\gamma$ t expression was detected in TCR $\beta$ <sup>lo/-</sup> CD1d- $\alpha$ GC<sup>-</sup> cells representing DP thymocytes, although CD4<sup>-</sup>CD8<sup>-</sup>TCR $\beta$ <sup>-</sup> cells did not express EGFP (Figure 2A left, and data not shown). However, CD1d- $\alpha$ GC<sup>+</sup> cells that were either CD4<sup>+</sup>8<sup>-</sup> or CD4<sup>-</sup>8<sup>-</sup>, which include both NK1.1<sup>-</sup> and NK1.1<sup>+</sup> cells, were negative for EGFP, suggesting that ROR $\gamma$ t is expressed exclusively in the DP thymocytes in TCR $\alpha\beta$  cells, but not in developing or mature iNKT cells (Figure 2A and data not shown). We then used ROR $\gamma$ t-cre transgenic mice as a DP thymocyte-specific deleter for another fate-mapping experiment (Figures 2B and 2C, and Figure S1). EGFP expression was also observed in the iNKT cells from thymus and liver of ROR $\gamma$ t-cre;ROSA26R mice. These data, together with the fate-mapping studies with CD4-cre transgenic mice, directly demonstrate that iNKT cells are progenies of DP thymocytes.

### Runx1 Is Cell Autonomously Required for iNKT Cell Development

We next examined the role of Runx1 in NKT cell development. Because Runx1 deficiency causes early em-

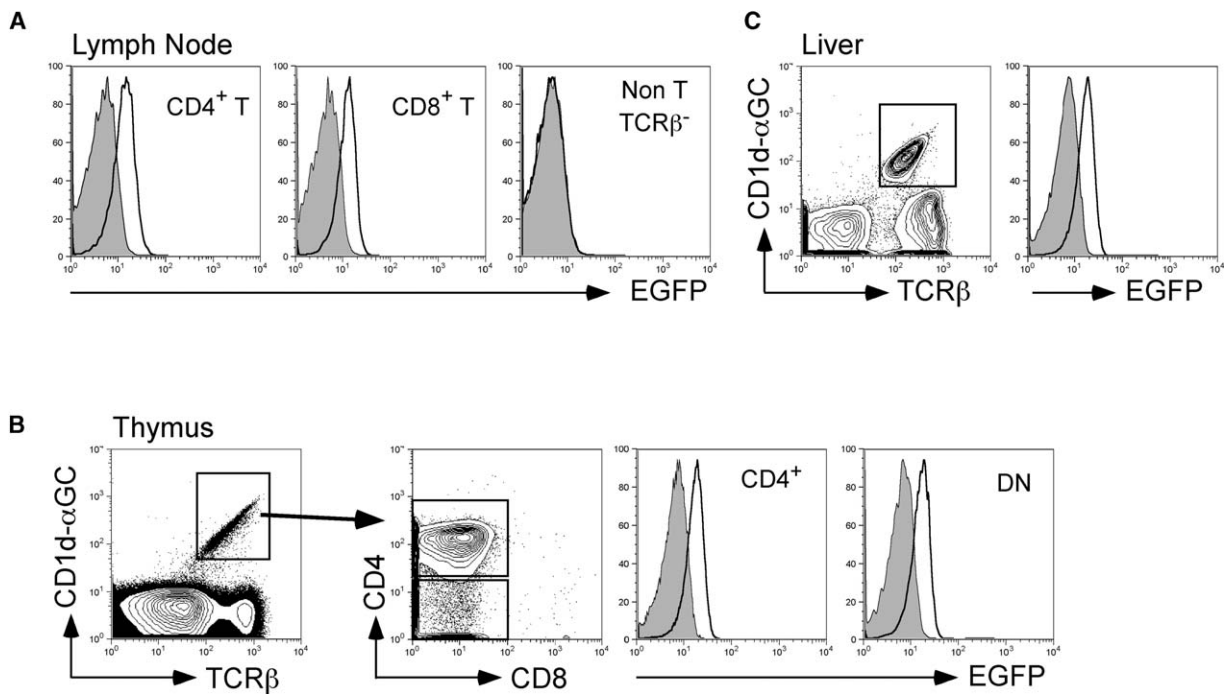


Figure 1. Fate Mapping for V $\alpha$ 14i NKT Cells with CD4-cre Mice

EGFP expression in peripheral TCR $\alpha\beta$  cells from lymph nodes (A) and iNKT cell populations in thymus (B) and liver (C) from ROSA26R mice (shaded histogram) and CD4-cre;ROSA26R (open histogram) is shown. V $\alpha$ 14i NKT cells were gated as indicated by rectangles, and EGFP expression in the gated iNKT cells is shown in the histograms. These data are representative of six different experiments.

embryonic lethality and complete lack of definitive hematopoiesis (Okuda et al., 1996), we used conditional gene targeting of *Runx1* in T cells to study its roles in iNKT cell development (Taniuchi et al., 2002). In this study, we bred mice harboring a loxP-flanked *Runx1* allele (*Runx1*<sup>f</sup>) to the CD4-cre mice to inactivate *Runx1* in DP thymocytes. Quantitative real-time polymerase chain reaction (Q-RT-PCR) analysis showed that the deletion efficiency in DP thymocytes was higher than 95% at the mRNA level (Figure S2). Development of DP thymocytes in CD4-cre;*Runx1*<sup>f/f</sup> mice was grossly normal (data not shown). However, CD1d- $\alpha$ GC tetramer<sup>+</sup> cells or TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup> cells were barely detected in thymus, spleen, and liver from CD4-cre;*Runx1*<sup>f/f</sup> mice, whereas approximately 0.2%–0.5% of thymocytes, 0.5%–1% of splenocytes, and 15% of liver lymphocytes were positive for TCR $\beta$  and CD1d- $\alpha$ GC tetramer in the littermate control (Figures 3A and 3B and data not shown). Co-staining with anti-NK1.1 and CD1d- $\alpha$ GC revealed that CD4-cre;*Runx1*<sup>f/f</sup> mice lacked NK1.1-CD1d- $\alpha$ GC<sup>+</sup> immature iNKT precursors (Figure 3C). Similar results were observed when *ROR $\gamma$ t-cre* was used for *Runx1* inactivation in DP thymocytes (data not shown). We used quantitative Taqman PCR to detect the invariant V $\alpha$ 14 to J $\alpha$ 18 TCR $\alpha$  transcript in total thymocytes or sorted DP thymocytes (Figures 3D and 3E). The relative amount of the invariant TCR $\alpha$  transcript, which presumably reflects the number of cells rearranging and expressing the V $\alpha$ 14i TCR $\alpha$  chain, was reduced by more than 10-fold in total thymocytes from CD4-cre;*Runx1*<sup>f/f</sup> mice compared to those from control CD4-cre or *Runx1*<sup>f/f</sup> littermates. However, the transcript levels in

sorted TCR $\alpha$ <sup>lo</sup>-CD4<sup>hi</sup>CD8<sup>hi</sup>CD1d- $\alpha$ GC<sup>-</sup> DP thymocytes, which represent developing thymocytes before positive selection, were similar between CD4-cre;*Runx1*<sup>f/f</sup> and control mice (Figure 3E). These findings indicate that in the absence of *Runx1* the canonical V $\alpha$ 14 to J $\alpha$ 18 rearrangement in DP thymocytes is not affected, although there is a profound reduction in the iNKT cells with this rearrangement in total thymus.

Development of iNKT cells requires expression of CD1d on DP thymocytes (Bendelac, 1995; Coles and Raulet, 2000). *Runx1* inactivation in DP thymocytes may therefore affect either iNKT cell precursors or the CD1d-expressing cells, thus altering antigen presentation. It is hence not clear whether the iNKT cell defect is cell autonomous or due to an altered thymic environment. Although there was no difference in CD1d expression on DP thymocytes between CD4-cre;*Runx1*<sup>f/f</sup> mice and littermate controls (data not shown), it is possible that antigen processing and presentation are affected, as is the case with saposin-deficient thymocytes (Kang and Cresswell, 2004; Zhou et al., 2004a). To address this issue, we examined iNKT cell development in mixed bone marrow chimeras in which irradiated mice were reconstituted with CD45.2<sup>-</sup> wild-type competitor and CD45.2<sup>+</sup> tester bone marrow cells from either CD4-cre;*Runx1*<sup>f/f</sup> or control CD4-cre mice. In the reconstituted mice, the contribution of CD45.2<sup>+</sup> tester cells to the DP compartment was approximately 50%, suggesting that both tester- and competitor-derived iNKT cells developed in almost identical environments (Figure 4A). In the chimeras prepared with control tester cells, iNKT cell development was detected both in CD45.2<sup>-</sup> and



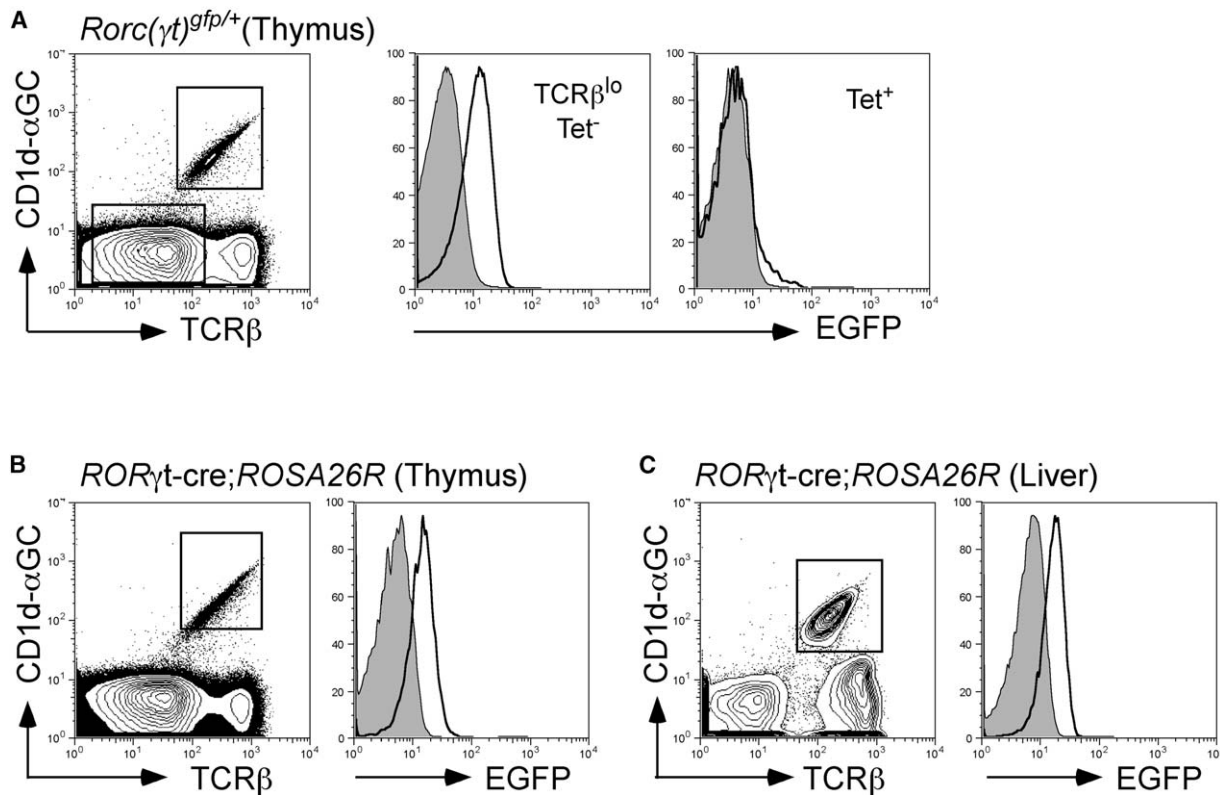


Figure 2. *ROR* $\gamma$ t Expression in NKT Cells and Fate Mapping with *ROR* $\gamma$ t-cre Mice

(A) *ROR* $\gamma$ t expression in thymocytes was detected with the knocked-in EGFP reporter in *Rorc*( $\gamma$ t)<sup>gfp/+</sup> mice. EGFP expression in TCR $\beta$ <sup>lo</sup>CD1d- $\alpha$ GC<sup>-</sup> and CD1d- $\alpha$ GC<sup>+</sup> gated thymocytes from *Rorc*( $\gamma$ t)<sup>gfp/+</sup> (open histograms) and wild-type littermate mice (shaded histograms) are shown. (B and C) EGFP expression in gated iNKT cells in thymus (B) and liver (C) from *ROSA26R* (shaded histogram) and *ROR* $\gamma$ t-cre;*ROSA26R* (open histogram) mice are shown.

CD45.2<sup>+</sup> compartments (Figure 4B). However, in the chimera reconstituted with CD45.2<sup>+</sup> *CD4-cre;Runx1*<sup>F/F</sup> bone marrow cells, there was barely any iNKT cell reconstitution with Runx1-deficient cells both in thymus and liver, whereas iNKT cells developed from CD45.2<sup>-</sup> competitor progenitor cells in the same hosts (Figure 4B). This result indicates that Runx1 is cell autonomously required for iNKT cell development.

#### Requirement for *ROR* $\gamma$ t and Bcl-xL in iNKT Cell Development

*ROR* $\gamma$ t prolongs the survival of DP thymocytes by upregulating transcription of Bcl-xL (Eberl et al., 2004; Kurebayashi et al., 2000; Sun et al., 2000). Because iNKT cells are derived from DP thymocytes, we next examined the requirement for *ROR* $\gamma$ t in iNKT cell development. To determine how loss of *ROR* $\gamma$ t in DP thymocytes affects iNKT cell development, we analyzed iNKT populations in thymus and liver from the *ROR* $\gamma$ t-deficient mice (*Rorc*( $\gamma$ t)<sup>gfp/gfp</sup>) and found that development of these cells was severely compromised. Almost no CD1d- $\alpha$ GC tetramer-positive cells, including NK1.1<sup>-</sup> immature precursors, were detected in thymus, spleen, and liver from *ROR* $\gamma$ t-deficient mice (Figure 5A and data not shown). Some NK1.1<sup>+</sup>TCR $\beta$ <sup>+</sup> cells were detected in thymus from *Rorc*( $\gamma$ t)<sup>gfp/gfp</sup> mice, but most of these

were CD4<sup>-</sup>CD8<sup>+</sup> and tetramer negative, suggesting that they were unlikely to be iNKT cells (Figure S3). Q-RT-PCR analysis showed that the level of the *V* $\alpha$ 14-*J* $\alpha$ 18 transcript in *ROR* $\gamma$ t-deficient thymocytes was approximately 1000-fold lower than that in wild-type control mice or 100-fold lower than in *CD4-cre;Runx1*<sup>F/F</sup> mice (Figure 5D), suggesting that *ROR* $\gamma$ t is required at an earlier stage than Runx1 for iNKT cell development. *ROR* $\gamma$ t-deficient thymocytes as well as Runx1-deficient thymocytes were capable of significantly stimulating a hybridoma expressing the *V* $\alpha$ 14-*J* $\alpha$ 18 invariant TCR $\alpha$  chain, indicating that the environment for iNKT cell development is not affected by the *ROR* $\gamma$ t deficiency (Figure S4). It was previously shown that forced expression of Bcl-xL in DP thymocytes rescues thymocyte development and promotes rearrangement of distal *V* $\alpha$  and *J* $\alpha$  segments in the absence of *ROR* $\gamma$ t (Eberl et al., 2004; Guo et al., 2002; Sun et al., 2000). Similarly, forced expression of a Bcl-xL transgene under the control of the *ROR* $\gamma$ t regulatory elements rescued the development of *V* $\alpha$ 14 iNKT cells (Figure 5B and data not shown). Similar numbers of CD1d- $\alpha$ GC tetramer-positive cells were detected in the thymus and liver from *Bcl-xL*<sup>tg</sup>;*Rorc*( $\gamma$ t)<sup>gfp/gfp</sup> mice and in the littermate *Bcl-xL*<sup>tg</sup>;*Rorc*( $\gamma$ t)<sup>gfp/+</sup> mice (Figure 5B and data not shown). Similar levels of *V* $\alpha$ 14-*J* $\alpha$ 18 transcript were also de-

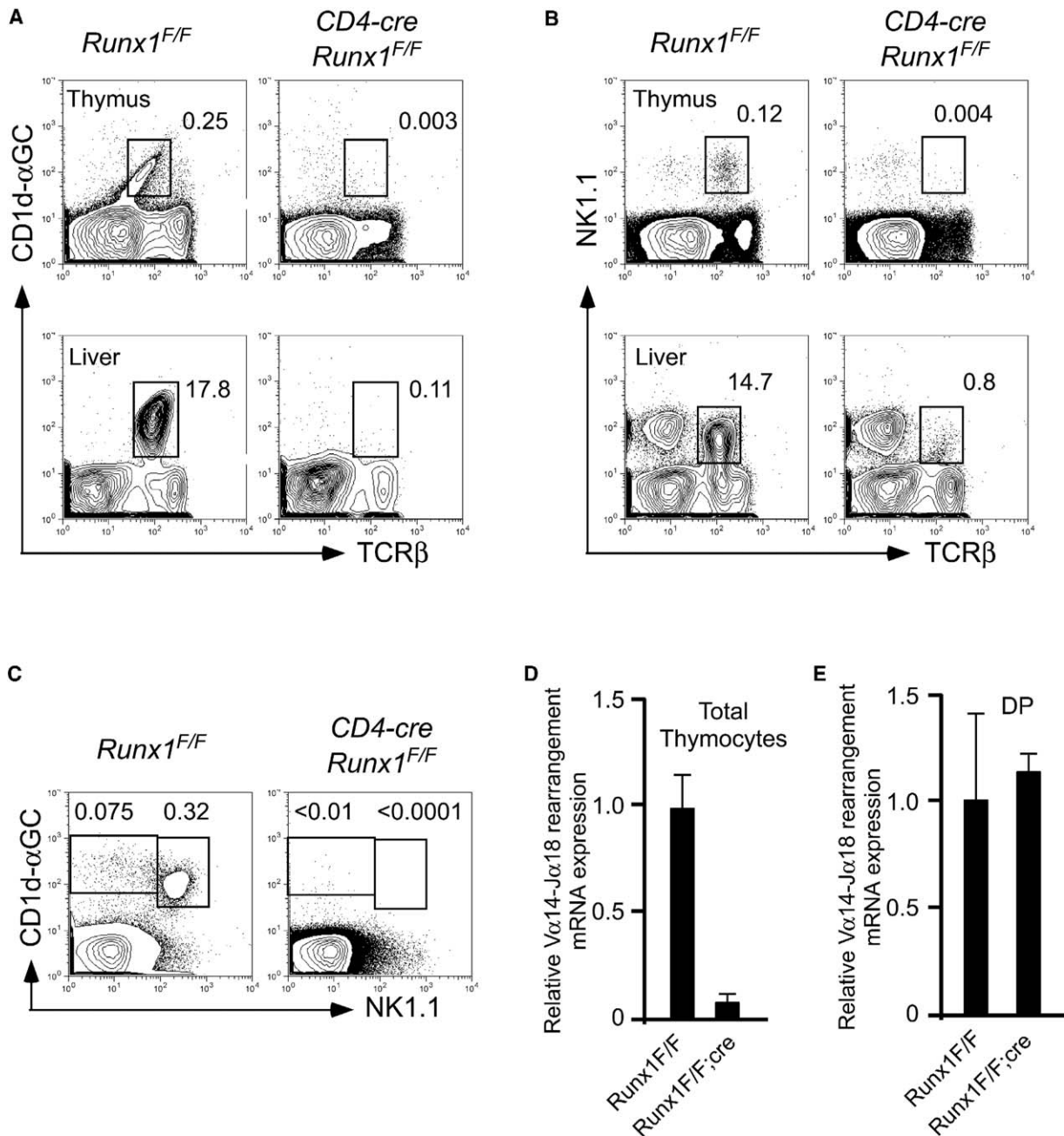


Figure 3. Runx1 Requirement in NKT Cell Development

(A and B) Flow cytometric analysis of NKT cell development in thymocytes (top) and liver (bottom) from *CD4-cre;Runx1<sup>F/F</sup>* and littermate *Runx1<sup>F/F</sup>* mice. Rectangles show gates for the NKT cell populations defined as CD1d- $\alpha$ GC<sup>+</sup> (A) or TCR $\beta$ <sup>+</sup>NK1.1<sup>+</sup> (B).

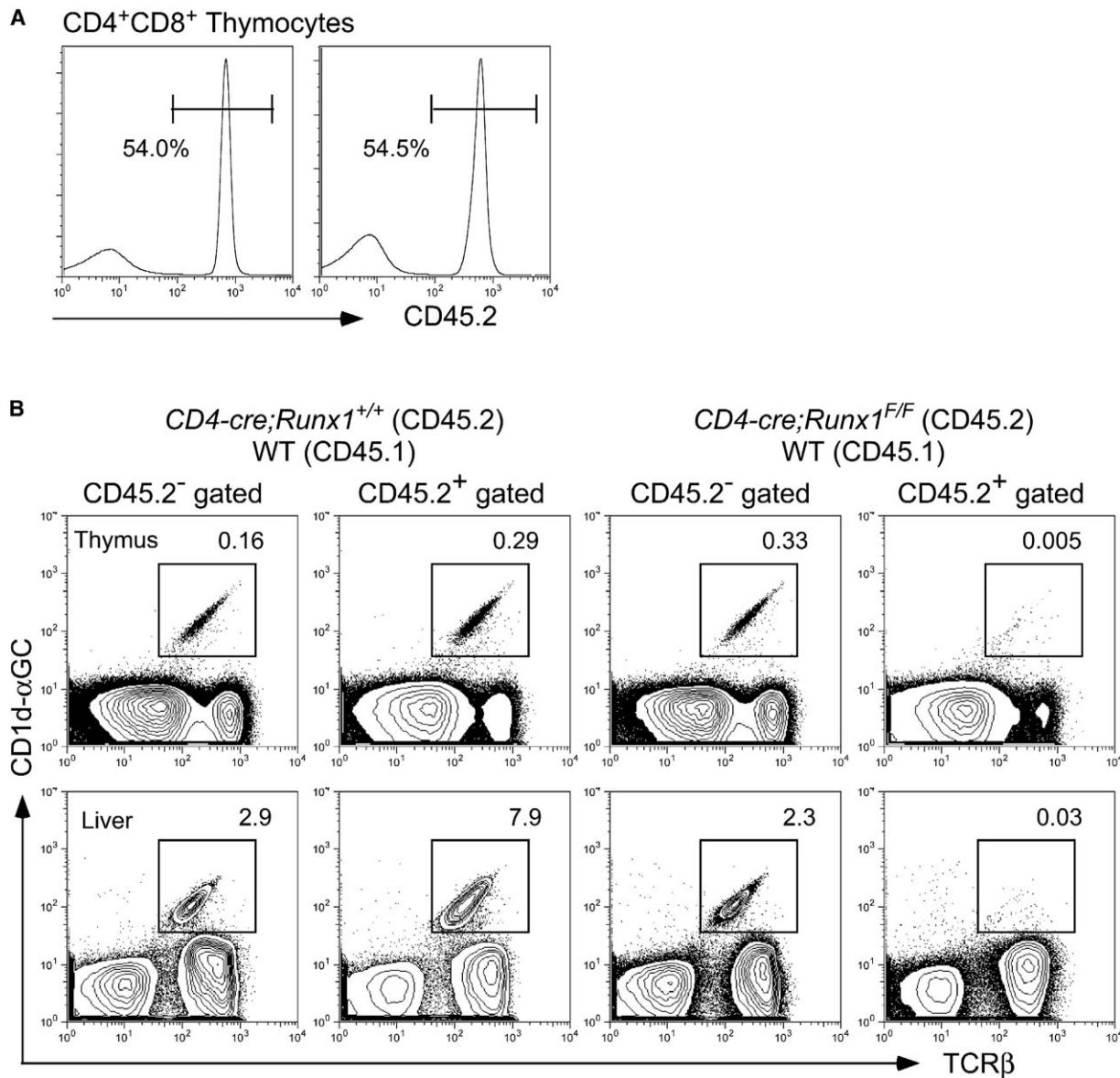
(C) Analysis of thymic iNKT cell precursors by costaining with CD1d- $\alpha$ GC tetramer and anti-NK1.1. Percentages of gated iNKT cell populations are shown.

(D and E) Real-time PCR quantification of the V $\alpha$ 14-J $\alpha$ 18 invariant TCR $\alpha$  transcript expression in total thymocytes (D) or sorted TCR $\beta$ <sup>lo/-</sup>CD1d- $\alpha$ GC<sup>-</sup>CD4<sup>hi</sup>CD8<sup>hi</sup> thymocytes (E). Levels of the invariant TCR $\alpha$  transcripts were normalized to those of C $\alpha$  transcripts. Results are shown as mean  $\pm$  SD from three independent experiments.

tested in *Rorc( $\gamma$ t)<sup>gfp/+</sup>* and *Rorc( $\gamma$ t)<sup>gfp/gfp</sup>* with the *Bcl-xL* transgene (Figure 5D). These results indicate that in the absence of ROR $\gamma$ t DP thymocytes fail to survive long enough to complete V $\alpha$ 14-J $\alpha$ 18 rearrangements.

iNKT cell development was also affected upon DP stage-specific inactivation of *Bcl-xL*. In *ROR $\gamma$ t-cre;Bcl-*

*xL<sup>F/F</sup>* mice, the number of CD1d- $\alpha$ GC-positive cells in thymus was reduced 5- to 10-fold compared to control mice (Figure 5C, top). Interestingly, the number of iNKT cells in liver was more severely affected in the absence of *Bcl-xL* (Figure 5C, bottom). The number of CD1d- $\alpha$ GC<sup>+</sup> NKT cells in liver from *ROR $\gamma$ t-cre;Bcl-xL<sup>F/F</sup>* was



**Figure 4. Cell-Autonomous Requirement for Runx1 in iNKT Cell Development**

Flow cytometric analysis of iNKT cell development in irradiated *Rag2*<sup>-/-</sup> host mice reconstituted with bone marrow from *CD4-cre;Runx1*<sup>F/F</sup> (CD45.2<sup>+</sup>) or control *CD4-cre* (CD45.2<sup>-</sup>) mixed with wild-type competitor (CD45.2<sup>-</sup>) bone marrow cells is shown.

(A) Contribution of CD45.2<sup>+</sup> *CD4-cre;Runx1*<sup>F/F</sup> (right) and control CD45.2<sup>+</sup> *CD4-cre* (left) bone marrow cells to CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes is shown.

(B) iNKT cell reconstitution in the thymus (top) and liver (bottom) in host mice prepared with CD45.2<sup>+</sup> *CD4-cre;Runx1*<sup>F/F</sup> or CD45.2<sup>+</sup> *CD4-cre* bone marrow cells was analyzed by flow cytometry. CD1d-αGC tetramer binding and anti-TCRβ staining in gated CD45.2<sup>+</sup> (tester) and CD45.2<sup>-</sup> (wild-type competitor) cells are shown. Percentages of gated iNKT cells are indicated. The data shown here are representative of four reconstituted animals.

reduced 50- to 80-fold compared to littermate controls. This finding may indicate that *Bcl-xL* is required for survival of iNKT cells in the periphery. Alternatively, *Bcl-xL* may be required late in intrathymic development of iNKT cells.

#### Fate Mapping of CD4<sup>-</sup>CD8<sup>-</sup>TCRβ<sup>+</sup> Thymocytes

Within the population of TCRαβ<sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> (DN-TCRαβ<sup>+</sup>) thymocytes, which include DP-derived iNKT

cells as shown above, there are cells whose developmental pathway remains undefined. We therefore examined the origin of these cells by fate mapping. EGFP expression was analyzed in gated DN-TCRαβ<sup>+</sup> CD1d-αGC<sup>-</sup> thymocytes from *RORγt-cre;ROSA26R* mice (Figure 6A). Among CD4<sup>-</sup>CD8<sup>-</sup> thymocytes, a large fraction of the cells were positive for TCRβ and negative for CD1d-αGC (Figure 6A). All of these cells were GFP<sup>+</sup>, whereas TCRβ<sup>-</sup> CD4<sup>-</sup>CD8<sup>-</sup> cells, which include imma-



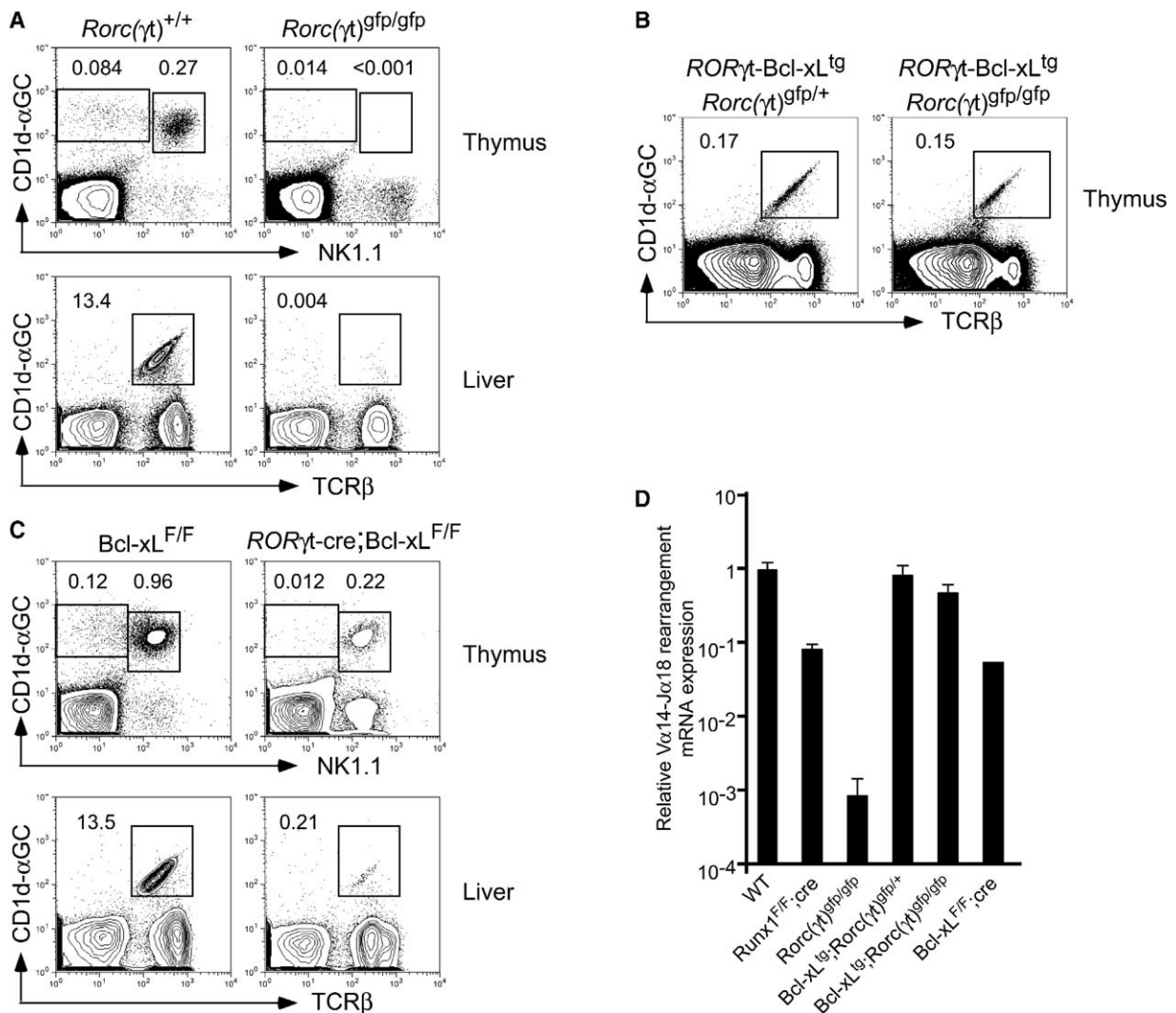


Figure 5. Requirement for ROR $\gamma$ t and Bcl-xL in iNKT Cell Development

(A) iNKT cell development in the thymus (top) and liver (bottom) of *RORγt*-deficient mice (*Rorc(γt)*<sup>gfp/gfp</sup>) was analyzed with anti-NK1.1 or anti-TCRβ and CD1d-αGC tetramer staining. Percentages of gated iNKT cell populations are shown. Data represent staining of cells from three different mice.

(B) Rescue of NKT cell development in *RORγt*-deficient mice by forced expression of Bcl-xL in DP thymocytes. CD1d-restricted NKT cells in the thymus from *RORγt*-deficient mice that express the *RORγt-Bcl-xL* transgene are shown. Data represent four different mice with the control littermates.

(C) Analysis of iNKT cell development in thymus (top) and liver (bottom) from mice with *RORγt-cre*-induced deletion of *Bcl-xL*. Data are shown as in (A).

(D) Real-time PCR quantification of the V $\alpha$ 14-J $\alpha$ 18 invariant *TCRα* transcript expressed in total thymocytes from mice with indicated genotypes: wild-type, n = 3; *CD4-cre;Runx1*<sup>F/F</sup>, n = 3; *Rorc(γt)*<sup>gfp/gfp</sup>, n = 3; *RORγt-Bcl-xL*<sup>tg</sup>; *Rorc(γt)*<sup>gfp/+</sup>, n = 4; *RORγt-Bcl-xL*<sup>tg</sup>; *Rorc(γt)*<sup>gfp/gfp</sup>, n = 4; *RORγt-cre;Bcl-xL*<sup>F/F</sup>, n = 2. Levels of the TCR transcripts were normalized to those of *Cα* transcripts. Results are shown as mean  $\pm$  SD.

ture thymocyte precursors and TCRγδ cells, did not express GFP. Similar results were observed in fate mapping with *CD4-cre* mice (data not shown). These data indicate that DN-TCRαβ<sup>+</sup> cells are selected from DP thymocytes.

In *RORγt*-deficient mice, in addition to the almost complete loss of double-negative iNKT cells, there was also a reduction in CD1d-αGC<sup>-</sup> DN-TCRαβ<sup>+</sup> cells (Figure 6B). This reduction was similar to that of mature, single-positive thymocytes (Figure 6C). The number of DN-TCRαβ<sup>+</sup> cells, similar to that of single-positive thy-

mocytes, was restored by expression of the *RORγt-Bcl-xL* transgene, suggesting that RORγt is unlikely to be specifically required for generation of DN-TCRαβ<sup>+</sup> thymocytes.

## Discussion

The pathway for development of V $\alpha$ 14i NKT cells has not been clearly defined (Kronenberg and Gapin, 2002; MacDonald, 2002). Although early studies proposed that the pathway is extrathymic (Makino et al., 1993),

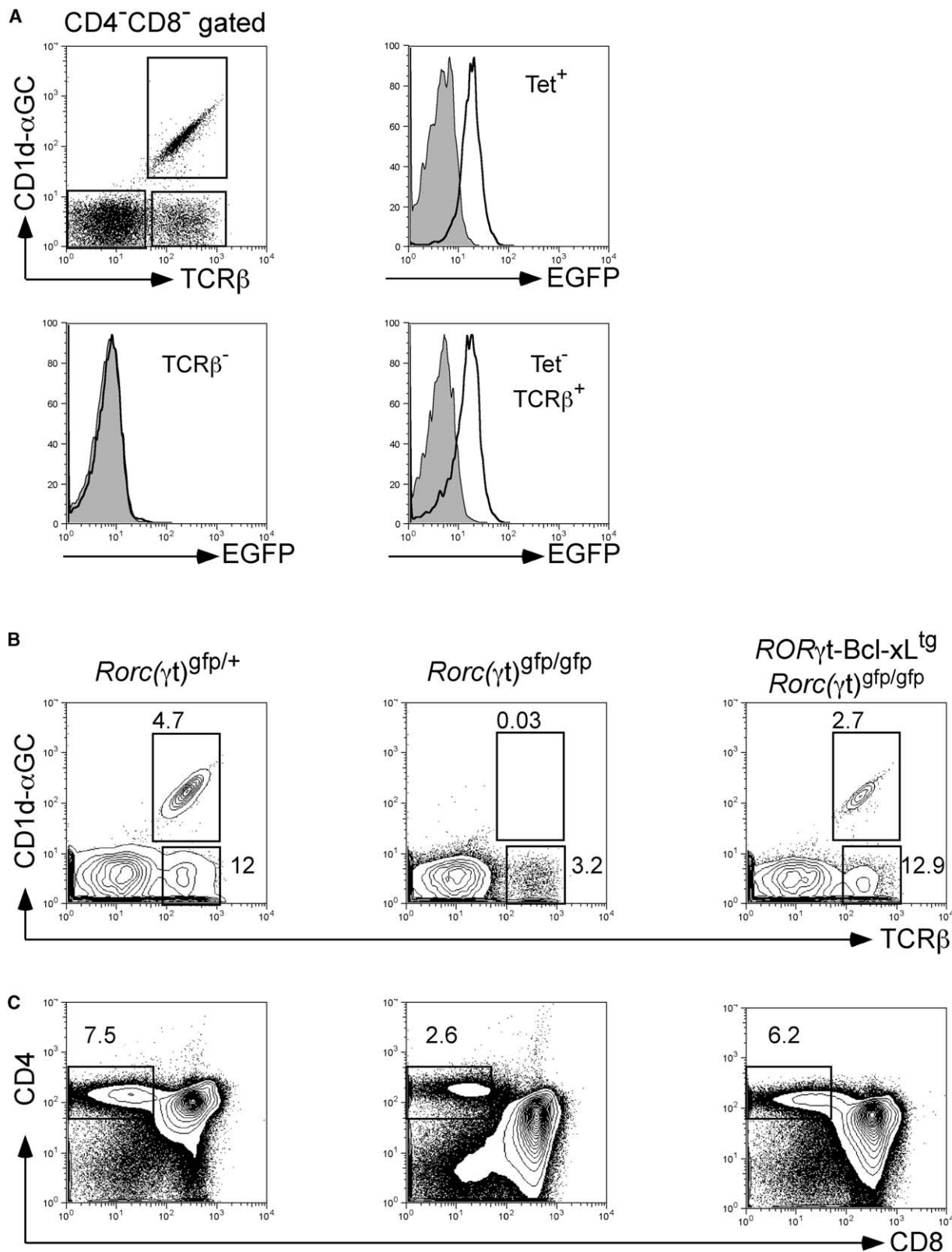


Figure 6. Fate Mapping of TCR $\alpha\beta$ <sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> Thymocytes

(A) EGFP expression in the CD4<sup>-</sup>CD8<sup>-</sup> thymocyte subpopulations from *ROSA26R* (shaded histogram) and *CD4-cre;ROSA26R* mice (open histogram) is shown. Expression of TCR $\beta$  and binding of CD1d- $\alpha$ GC tetramer in gated CD4<sup>-</sup>CD8<sup>-</sup> thymocytes are indicated by the gating rectangles. EGFP expression in the gated populations is shown as histograms. These data are representative of six different experiments. (B and C) Development of TCR $\alpha\beta$ <sup>+</sup> CD4<sup>-</sup>CD8<sup>-</sup> populations (B) and CD4 single-positive mature thymocytes (C) from wild-type (left), *ROR* $\gamma$ t-deficient mice (middle), and *ROR* $\gamma$ t-deficient mice that express the *ROR* $\gamma$ t-Bcl-xL transgene (right). Percentages of gated populations are shown.



subsequent bone marrow transplantation experiments with thymectomized recipients demonstrated that iNKT cell development is thymus dependent (Tilloy et al., 1999), and it was further suggested, but not firmly established, that DP thymocyte precursors are involved (Gapin et al., 2001). Two models have been proposed for how iNKT cells develop in the thymus: a precommitment model, in which iNKT cells adopt a distinct lineage early in thymocyte differentiation, and a selection model, in which thymocytes with V $\alpha$ 14-J $\alpha$ 18 rearrangements are positively selected from the large pool of conventional DP thymocytes after random TCR $\alpha$  rearrangements. In this paper, we have used fate mapping and analysis of mice with mutations in two key transcription factors to demonstrate that iNKT cells are derived from DP thymocytes and to provide evidence that is most consistent with the selection model for iNKT cell development.

Cre-mediated cell fate mapping is a powerful tool for in vivo analysis of development of specific cell lineages and is especially useful because it avoids perturbing normal development, which can occur when irradiation and adoptive transfer are used to study hematopoietic lineages. In this study, we used two different Cre transgenic strains to firmly demonstrate that all V $\alpha$ 14i NKT cells, including those that are NK1.1<sup>-</sup> and CD4<sup>-</sup>8<sup>-</sup>, are derived from DP thymocytes. *ROR $\gamma$ t-cre* expression is specific to DP thymocytes, whereas *CD4-cre* is expressed more broadly in DP cells as well as CD4<sup>+</sup> T cells and probably also CD4<sup>+</sup> NKT cells. Because the CD1d- $\alpha$ GalCer<sup>+</sup> cells were genetically labeled after crossing both Cre transgenic strains to the ROSA26-GFP reporter strain, we can conclude that all CD1d-dependent NKT cells are thymus dependent and pass through the DP stage.

Analyses of the requirements for ROR $\gamma$ t and Runx1 in iNKT cell development further buttress our conclusion that these cells are derived from DP thymocytes. Mice lacking ROR $\gamma$ t, which is expressed exclusively in DP thymocytes during T cell development, had essentially no iNKT cells and no V $\alpha$ 14-J $\alpha$ 18 TCR $\alpha$  rearrangements in thymocytes. Mice in which Runx1 was inactivated solely in DP thymocytes likewise lacked iNKT cells, despite normal levels of V $\alpha$ 14-J $\alpha$ 18 in DP cells. Together with demonstration that both ROR $\gamma$ t and Runx1 are required cell autonomously in iNKT cell precursors, these results establish that iNKT cells differentiate through a DP-intermediate stage.

The differences in canonical TCR $\alpha$  rearrangements in mice deficient for ROR $\gamma$ t and Runx1 suggest that these transcription factors function at different stages of iNKT cell development and provide insight into the mechanism of commitment to the iNKT cell lineage. We previously showed that ROR $\gamma$ t is dispensable for rearrangement of 3' V $\alpha$  and 5' J $\alpha$  segments that are located most proximally to each other in the TCR $\alpha$  locus (Guo et al., 2002). However, rearrangement of more distal segments requires ROR $\gamma$ t or expression of its downstream target, Bcl-xL. V $\alpha$ 14-J $\alpha$ 18 rearrangements, which involve distal segments, were absent in the ROR $\gamma$ t-deficient mice but were rescued, along with iNKT cell development, upon forced expression of Bcl-xL selectively in DP thymocytes. These results are most consistent with a model in which prolonged survival of

DP thymocytes favors successive V $\alpha$ -J $\alpha$  rearrangements until thymocytes receive a strong positive-selection signal. TCRs composed of products of V $\alpha$ 14-J $\alpha$ 18 rearrangements would thus be stochastically generated and rapidly selected by interaction with CD1d and endogenous lipid ligand. The selection of these DP cells appears to result in rapid loss of expression of CD8 and, often, CD4, as it has not been possible to detect CD1d- $\alpha$ GC tetramer-positive DP thymocytes. The presence of normal levels of V $\alpha$ 14-J $\alpha$ 18 rearrangements, despite complete absence of CD1d- $\alpha$ GC tetramer-positive cells, after *Runx1* inactivation in DP thymocytes suggests that this transcription factor is required after rearrangement is complete and during selection or subsequent expansion of iNKT precursor cells.

Our results suggest that the same mechanism of stochastic rearrangement within the TCR $\alpha$  locus occurs in the generation of iNKT cells and conventional thymocytes. Thus, CD1d-reactive iNKT cell precursors are most likely selected from the same pool of ROR $\gamma$ t/Bcl-xL-dependent DP thymocytes as mainstream T cells that commit to the helper and cytotoxic lineages. In favor of such a process is the finding that a V $\alpha$ 14-J $\alpha$ 18 TCR transgene rescued iNKT cell development in ROR $\gamma$ -deficient mice (Bezbradica et al., 2005). Together with earlier evidence that rearrangements of the silent TCR $\alpha$  allele in iNKT cells are not canonical (Shimamura et al., 1997), these results argue against a directed rearrangement of V $\alpha$ 14 to J $\alpha$ 18 segments, which is central to a precommitment model of iNKT cell development.

Because iNKT cells failed to develop in the absence of Runx1 despite normal levels of V $\alpha$ 14-J $\alpha$ 18 rearrangements at the DP stage, this transcription factor appears to be required during selection and/or further maturation of this T cell lineage. In contrast, we observed only a modest reduction in selection and intrathymic development of conventional CD4 and CD8 lineage T cells when Runx1 was inactivated in DP thymocytes (T.E., I.T., and D.R.L., unpublished data). This may reflect different requirements in signaling by TCRs on iNKT lineage cells versus conventional T cells. Alternatively, this may be related to the requirement for iNKT cell precursors, unlike conventional CD4- and CD8-lineage T cells, to undergo multiple divisions within the thymus after selection (Benlagha et al., 2002). This may allow more rapid dilution of Runx1 protein after Cre-mediated gene inactivation. Nevertheless, several other factors are selectively required for intrathymic development of iNKT cells, but not conventional T cells, suggesting that there are fundamental differences in signaling through TCR and costimulatory receptors during selection of these distinct lineages (Kronenberg and Gapin, 2002). For example, the TCR-proximal tyrosine kinase Fyn and intact NF- $\kappa$ B signaling are required for iNKT cell, but not conventional T cell, development (Eberl et al., 1999; Schmidt-Suppran et al., 2004; Sivakumar et al., 2003; Stanic et al., 2004a, 2004b). NF- $\kappa$ B may be required relatively late in iNKT cell development to promote survival of proliferating cells, as transgenic expression of Bcl-xL restores development of iNKT cells in mice with defective NF- $\kappa$ B activation (Stanic et al., 2004a). Mice deficient for both IL-7 and IL-15 displayed a deficiency of iNKT cell precursors as severe as that

observed in the absence of Runx1 (Matsuda et al., 2002). Runx1 could potentially regulate expression of receptors for these cytokines or for components of their downstream signaling pathways in iNKT cell precursors.

TCR $\alpha\beta$  signals during positive selection activate downstream signaling cascades to allow DP thymocytes to differentiate into a variety of mature T cells, including conventional T cells, iNKT cells, regulatory T cells, and intraepithelial lymphocytes (IEL). Positive selection of iNKT cells results in cell proliferation and expression of NK-related genes. These features are specific to the iNKT lineage and are probably tightly related to commitment to the iNKT cell lineage. iNKT cells are also likely to express chemokine receptors that target their migration to liver. These unique features suggest that invariant TCRs, upon interaction with CD1d during selection of iNKT cells, transduce signals that induce distinct gene expression. It has been suggested that iNKT cells are autoreactive and are selected by strong agonist signals at the boundary between positive and negative selection (Bendelac, 2004). CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and gut TCR $\alpha\beta$  CD8 $\alpha\alpha$ <sup>+</sup> IEL, which are derived from DP thymocytes and express NK1.1, are also autoreactive and selected through agonistic selection signals, although it has not been clearly demonstrated whether these types of cells proliferate after positive selection like iNKT cells (Bendelac, 2004). Thus, it is likely that strong agonist signals can induce differentiation to nonconventional T cells, including iNKT cells. This hypothesis is supported by studies with TCR transgenic systems showing that selection of thymocytes by self-antigens induces expression of NK1.1 and other NK-related genes even in the absence of invariant TCR rearrangements (Legendre et al., 1999; Yamagata et al., 2004). These studies and another study also showed that agonistic TCR stimulation results in emergence of DN-TCR $\alpha\beta$ <sup>+</sup> thymocytes, whose origins were not clearly demonstrated, as well as mature thymocytes expressing CD8 $\alpha\alpha$  homodimer (Legendre et al., 1999; Leishman et al., 2002; Yamagata et al., 2004). It has been suggested that DN-TCR $\alpha\beta$ <sup>+</sup> thymocytes are precursors for TCR $\alpha\beta$  CD8 $\alpha\alpha$ <sup>+</sup> IEL (Gangadharan and Cheroutre, 2004; Guy-Grand et al., 2001). Our fate-mapping studies clearly show that the DN-TCR $\alpha\beta$ <sup>+</sup> cells are derived from DP thymocytes. It remains unclear, however, if these cells are precursors of TCR $\alpha\beta$  CD8 $\alpha\alpha$ <sup>+</sup> IEL or if all such IEL are derived from CD8 single-positive thymocytes. It will be important to determine if agonistic selection is involved in differentiation of non-TCR transgenic thymocytes and if it requires a distinct set of self-antigens.

Conditional inactivation of *Bcl-xL* at the DP thymocyte stage resulted in the reduction of iNKT cell numbers in both thymus and liver. The reductions of iNKT cells and V $\alpha$ 14-J $\alpha$ 18 rearrangements were milder than in ROR $\gamma$ t-deficient mice. With the ROR $\gamma$ t-*cre* transgene, *Bcl-xL* deletion is initiated in early DP thymocytes, and there may be some delay between DNA deletion and protein loss, as is often the case with other conditional knockout mice. This delay is likely to allow a fraction of DP thymocytes to undergo TCR $\alpha$  rearrangements of distal V $\alpha$  and J $\alpha$  segments, including V $\alpha$ 14 to J $\alpha$ 18, and would give rise to V $\alpha$ 14i NKT cells. Reduction of liver

iNKT cells was more remarkable in the absence of Bcl-xL compared to iNKT cells in the thymus. In contrast, the number of conventional TCR $\alpha\beta$  cells in the periphery was normal (data not shown). This implies that Bcl-xL-deficient iNKT cells have a defect late in maturation or in survival after migrating to the periphery. Because ROR $\gamma$ t is not expressed in selected iNKT cells, the Bcl-xL requirement thus appears to have both ROR $\gamma$ t-dependent and -independent components during iNKT cell development and homeostasis.

Taken together, the data presented in this study provide genetic evidence that iNKT cells develop through the DP thymocyte stage. Findings from analyses of mutant mice support the model that iNKT cells are selected from a common pool of DP thymocytes undergoing random TCR $\alpha$  rearrangements. We also showed nonredundant requirements of Runx1 and ROR $\gamma$ t at different stages of iNKT cell development. It is likely that Runx1 is involved in selection or survival of selected intermediate precursors for iNKT cells, whereas ROR $\gamma$ t supports survival of the common precursor pool during the TCR $\alpha$  rearrangements. Studies on molecular mechanisms involved in the Runx1 requirement may provide further insights into the mechanisms that distinguish commitment of different mature T cell populations from a commonly shared precursor pool of DP thymocytes.

## Experimental Procedures

### Mice

ROR $\gamma$ t-*cre* mice, *CD4-cre* mice, ROR $\gamma$ t-deficient mice, ROR $\gamma$ t-Bcl-xL transgenic mice, *Runx1* conditional knockout mice, Bcl-xL conditional knockout mice, and ROSA26-EGFP-*cre* reporter mice were described previously (Eberl and Littman, 2004; Eberl et al., 2004; Mao et al., 2001; Rucker et al., 2000; Sun et al., 2000; Taniuchi et al., 2002). All of these mice have been backcrossed to the C57BL/6 background. *CD4-cre;Runx1<sup>F/F</sup>* mice used for transplantation experiments had been backcrossed into the C57BL/6 background more than seven times. Mouse colonies were maintained in a specific, pathogen-free facility in the Skirball Institute, and experiments were performed according to the approved protocol by the Institutional Animal Care and Use Committee.

### Flow Cytometry

Liver lymphocytes were separated by 40% and 80% Percoll (Amersham) gradient centrifugation. Cells were stained with monoclonal antibodies anti-CD4 (GK1.5 or RM4-5), anti-CD8 (53.6.7), anti-CD45.2 (104), anti-TCR $\beta$  (H57-597), anti-NK1.1 (PK136), and anti-B220 (RA3-6B2) (BD Pharmingen, San Diego, CA). For CD1d- $\alpha$ GC tetramer staining, cells were preincubated with unconjugated streptavidin (Sigma) and anti-CD16/32 (2.4G2, BD Pharmingen) for 15 min on ice and then stained with a mixture of fluorescently labeled CD1d- $\alpha$ GC and monoclonal antibodies for 20 min at room temperature. Stained cells were analyzed with an LSRII cytometer (BD Immunocytometry Systems, San Jose, CA) or sorted with a MoFlow (DAKO Cytomation, CO). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

### Bone Marrow Transplantation

Bone marrow cells were harvested from CD45.2<sup>+</sup> *CD4-cre;Runx1<sup>F/F</sup>* mice, littermate *CD4-cre;Runx1<sup>+/+</sup>* mice (CD45.2, tester), and wild-type CD45.1 C57BL/6 mice (competitor). 1:1 mixtures of tester and competitor cells were transferred into lethally (950 rad) irradiated *Rag2<sup>-/-</sup>* (CD45.2, C57BL/6 background) mice. Reconstituted NKT cell populations were analyzed 10–14 weeks after transfer.

### Quantitative RT-PCR

RNA and first-strand cDNA were prepared with Trizol and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according

to the manufacturer's instructions. Q-RT-PCR was performed with iCycler (Bio-Rad, Hercules, CA) and Quantitect Probe PCR kit (QIAGEN, Valencia, CA), with primers and probes previously described (Gapin et al., 2001; Townsend et al., 2004).

#### Antigen Presentation Assay

5 × 10<sup>5</sup> thymocytes from *CD4-cre;Runx1<sup>F/F</sup>* or *Rorc(γt)<sup>gfp/gfp</sup>* mice were incubated with 5 × 10<sup>4</sup> DN32D3 cells for 24 hr, and IL-2 content in the supernatant was measured with the CTLL-2 cell line as previously described (Zhou et al., 2004a).

#### Supplemental Data

Supplemental Data including four figures are available online with this article at <http://www.immunity.com/cgi/content/full/22/6/705/DC1/>.

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